Binding Surface in Zβ Domain from Human ZBP1 Does Not Require Conserved Proline Residues for Z-DNA Binding and B-to-Z-DNA Conversion Activities

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Z-DNA binding protein 1 (ZBP1; also known as DLM-1 and DAI), a tumor associated protein, is activated by various forms of cellular stresses such as tumorigenesis and viral infections,[1] and includes complex transcription variants by the result of alternative splicings.[2] As the name implies, ZBP1 possesses two Z-DNA binding domains, Zα_{ZBP1} and Zβ_{ZBP1}, at its N-terminus.[2,3] Previously we showed that both hZα_{ZBP1} and hZβ_{ZBP1} bind Z-DNA and exhibit B-to-Z conversion activity.[4] Recent studies demonstrated that hZα_{ZBP1} domain is also involved in subcellular localization of human ZBP1.[5] ZBP1 has been alternatively named DAI (DNA-dependent activator of IFN-regulatory factors) since a recent study revealed that its plays a role as a cytosolic DNA sensor in the innate immune response.[6]

The Z-DNA binding domain Zα was originally identified from human ADAR1 (double strand RNA adenosine deaminase 1) and has been subsequently found in more members of proteins from various organisms.[7,8] Vertebrate proteins including ADAR1 and ZBP1 as well as a recently identified protein kinase PKZ[9] have two tandem Zα domains, referred to Zα and Zβ, respectively, at their N-termini. In contrast, the E3L protein of vaccinia virus and its orthologs in poxviruses possess only one Zα domain at their N termini.[10] It has been shown that these Zα domains bind strongly and specifically to Z-DNA.[4,7,9,11-16]

While Z-DNA binding appears to be a common feature for the Zα domains, variations at amino acid sequence level have been recognized. Sequence and structural analyses revealed that several residues of Zαs are critical for Z-DNA binding activity. They are highly conserved and their mutations often result in drastically reduced Z-DNA binding activity.[17] Among those conserved amino acid residues, the tyrosine in the helix α3 is the most critical. For instance, the human Zβ_{ADAR1} has no Z-DNA specific binding activity because of lacking a tyrosine residue in the helix α3,[18] while in other Zβ domains from zebra fish ADAR1, ZBP1 and PKZ, this key residue is highly conserved (Fig. 1). Also important is highly conserved proline residue(s) in the C-

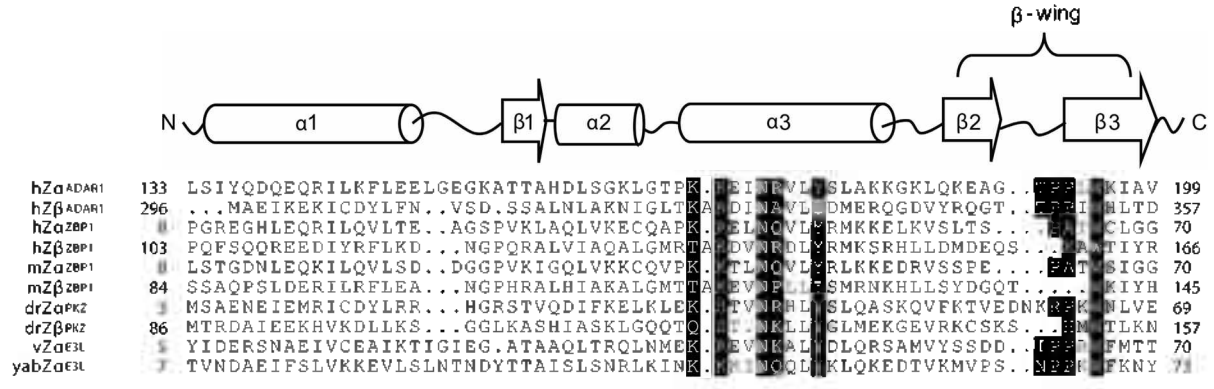

Figure 1. Secondary structures and sequence alignment of Zβ_{ZBP1} and other Z-DNA binding domains. Secondary structural elements are depicted above the alignment. Starting and ending residue numbers are shown. Residues interacting with Z-DNA identified by the crystal structures of human Zα_{ADAR1} (hZα_{ADAR1}), murine Zα_{ZBP1} (mZα_{ZBP1}) and yaba-like disease virus Zα_{E3L} (yabZα_{E3L}) are marked in black boxes.[19-21] The residues interacting with Z-DNA are located in the helix α3 and the β-wing region (β-wing). Human Zβ_{ADAR1}, which lacks the key tyrosine in the helix α3 (α3), does not bind to Z-DNA because of the lacking tyrosine is replaced by isoleucine (highlighted in gray box). The human and mouse Zβ_{ZBP1}s both lack proline(s) that is highly conserved in other Zαs except for the second Z-DNA binding domain (Zβ) of PKZs. GenBank accession numbers for the various Zα protein domain sequences are as follows: double-stranded RNA adenosine deaminase 1 (*Homo sapiens*): AAB06697; Z-DNA binding protein 1 (*Homo sapience*): Q9H171; Z-DNA-binding protein 1 (*Mus musculus*): NP_067369; Z-DNA binding protein kinase (*Danio rerio*): CAG25513; E3L protein (vaccinia virus): AAA02759; E3L protein (yaba-like disease virus): NP_073419.

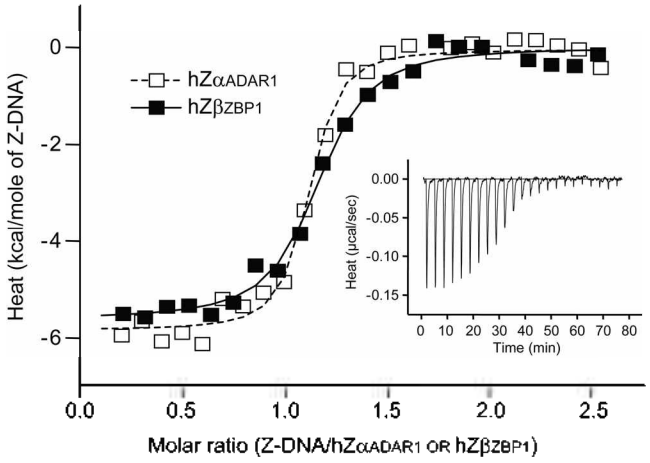

Figure 2. *In vitro* binding of $d(CG)_3$ to $hZ\beta_{ZBP1}$. ITC was used to analyze binding of $hZ\beta_{ZBP1}$ mutants to $d(CG)_3$ in solution. Main graphs show representative curves fitted to one-site model for $hZ\alpha_{ADAR1}$ (open square with dashed line) and $hZ\beta_{ZBP1}$ wild type (filled square with solid line) along with the data. Inserts display raw heat change elicited by successive injections of $d(CG)_3$ to $hZ\beta_{ZBP1}$ wild type.

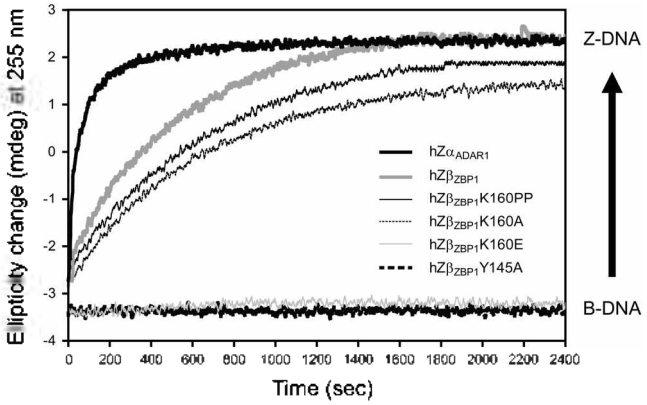

Figure 3. Kinetics of B-to-Z conversion by $hZ\beta_{ZBP1}$ and its mutants. The ability of $hZ\beta_{ZBP1}$ mutants to convert $d(CG)_6$ duplex from B-DNA to Z-DNA is monitored by circular dichroism. The ellipticity changes at 255 nm are shown as a function of time. $hZ\alpha_{ADAR1}$ (thick and black) converted the substrate DNA to the Z-DNA conformation quite rapidly, while wild-type $hZ\beta_{ZBP1}$ (thick and grey) showed such conversion at a slower rate. $hZ\beta_{ZBP1}$:K160PP (thin and black) showed a slightly decreased activity and $hZ\beta_{ZBP1}$:K160A (thin and dotted) exhibited an even more reduced activity. Note that $hZ\beta_{ZBP1}$:K160E (thin and grey) and $hZ\beta_{ZBP1}$:Y145A (thick and dotted) almost completely lost B-to-Z conversion activity.

terminal β-wing region of the $Z\alpha$ domains (Fig. 1). This conserved proline(s) interacts with Z-DNA backbone through van der Waals interaction(s).[19-21] Interestingly, such proline(s) is missing in the C-terminal region of the $Z\beta$ domains from ZBP1 and PKZ proteins, suggesting a possible structural variation for Z-DNA binding mode of the $Z\beta$ domains.

To investigate the roles of the conserved residues in the $Z\alpha$ domains for human $Z\beta_{ZBP1}$ ($hZ\beta_{ZBP1}$) activities, we performed mutational analysis and examined their effects on Z-DNA binding by isothermal titration calorimetry (ITC) (Fig. 2 and Table 1) and on B-to-Z conversion by circular dichroism (CD) (Fig. 3). ITC was used to analyze binding affinities between $Z\alpha$s and Z-DNA. CD has been shown to be powerful in examining B-to-Z conversion activity of $Z\alpha$s.[22]

ITC measurements were carried out to determine apparent dissociation constants (K_D) of $hZ\beta_{ZBP1}$ and other $Z\alpha$s to their cognate DNA sequence. The substrate DNA used in this study was double-stranded $d(CG)_3$, a minimal sequence known to contain two binding sites for $Z\alpha$ – one for each DNA strand.[19-21] The results from a typical ITC experiment are illustrated in Figure 2 and K_Ds of various $Z\alpha$s were summarized in Table 1. As demonstrated in Figure 2, data fitted well to curves calculated from a 1:1 stoichiometry model (one protein per each DNA strand). With a $Z\alpha$ protein or the substrate DNA alone, ITC signals were similar to those for buffer, indicating that no non-specific binding occurred (data not shown). Based on the ITC data, $hZ\beta_{ZBP1}$ showed somewhat weaker affinity compared to that of $hZ\alpha_{ADAR1}$ while $hZ\alpha_{ZBP1}$ binds the DNA as strongly as $hZ\alpha_{ADAR1}$. This weaker affinity of $hZ\beta_{ZBP1}$ could have resulted from sequence variation and more possibly from the lacking proline in the putative β-wing region of $hZ\beta_{ZBP1}$.

Table 1. Binding affinities of investigated $Z\alpha$s and $hZ\beta_{ZBP1}$ mutants with $d(CG)_3$ duplex determined by ITC

Protein	Binding constant (K_D) (nM)
$Z\alpha_{ADAR1}$	31.1
$Z\alpha\beta_{ZBP1}$	34.3
$Z\alpha_{ZBP1}$	38.7
$Z\beta_{ZBP1}$:WT[a]	83.3
$Z\beta_{ZBP1}$:K160A[b]	100
$Z\beta_{ZBP1}$:K160P	121
$Z\beta_{ZBP1}$:K160E	165
$Z\beta_{ZBP1}$:Y145A	309

[a]WT, wild-type. [b]Single-letter amino acid nomenclature is used.

To examine the importance of the residues in the helix $\alpha 3$ and the putative β-wing of $hZ\beta_{ZBP1}$, we prepared the following mutants: Y145A, K160A, K160PP and K160E (single-letter amino acid nomenclature was used). ITC analysis for these mutants revealed that all the $hZ\beta_{ZBP1}$ mutants show similar binding stoichiometry but lower binding affinities compared to that of the wild-type $hZ\beta_{ZBP1}$ (Table 1). In particular $hZ\beta_{ZBP1}$:Y145A exhibited an almost four-fold higher K_D than that of the wild type, indicating its weaker affinity for Z-DNA. Such decrease in K_D by Y145A was expected because of the importance in Z-DNA binding and the degree of conservation of Y145 among $Z\alpha$s. All known crystallographic structures of $Z\alpha$s further support the crucial role of Y145 in Z-DNA binding.[19-21]

$hZ\beta_{ZBP1}$:K160 mutants showed weaker binding affinities with varied degrees, suggesting the importance of K160 for Z-DNA binding. Accumulating evidence indicates that all

Zαs structurally characterized so far exhibit an α/β architecture consisting of three β strands and three α helices.[19-21,23] The helices α2 and α3 form a helix-turn-helix (HTH) motif and two anti-parallel β strands (β2 and β3) form a so-called β-wing region that is important for Z-DNA recognition.[19-21] The high degree of conservation of the proline in the β-wing among Zα family members also underlines the importance of the β-wing. However, this conserved proline residue is absent and substituted by a lysine (K160) in hZβ_{ZBP1} (Fig. 1).

B-to-Z conversion activities of hZβ_{ZBP1} mutants were examined by CD with double-stranded d(CG)$_6$. hZβ_{ZBP1}:Y145A showed no B-to-Z conversion activity, again confirming the importance of Y145 in the helix α3 of hZβ_{ZBP1} (Fig. 3). When K160 was mutated to the tandem prolines, the resulting protein hZβ_{ZBP1}:K160PP exhibited a slightly decreased B-to-Z conversion rate compared with that of the wild type. The rate of B-to-Z conversion was further decreased in hZβ_{ZBP1}:K160A. Consequently both mutants demonstrate less complete B-to-Z conversion activities as well (Fig. 3). The decreases in the B-to-Z conversion activity by K160PP and K160A mutants may have been caused by losing the positively charged side chain of K160. This explanation was further supported by another parallel mutant hZβ_{ZBP1}:K160E where lysine was mutated to glutamate, thereby reversing the charge of the residue.

The hZβ_{ZBP1}:K160E, possessing a negatively charged side chain opposite of the wild-type hZβ_{ZBP1}, almost eliminated the B-to-Z conversion activity (Fig. 3). This is another example that can effectively demolish the protein activity by single point mutation as witnessed in hZβ_{ZBP1}:Y145A. This result strongly suggested that the positive charge from hZβ_{ZBP1}:K160 interact directly with Z-DNA by forming charge-to-charge interaction between the negatively charged phosphate backbone of Z-DNA and the positive charge from hZβ_{ZBP1}:K160. It is plausible that the putative β-wing region of hZβ_{ZBP1} lacking proline could be still involved in Z-DNA binding but interacting in a different mode. In the co-crystal structure of hZα_{ADAR1}/Z-DNA, P193 corresponding to K160 of hZβ_{ZBP1} positions very close to Z-DNA and forms van der Waals interaction with Z-DNA to stabilize their interaction.[19] In contrast, hZβ_{ZBP1} may use a relatively long side chain with a positive charge in the β-wing to enhance its interaction with Z-DNA.

Zα domains appear to recognize and bind Z-DNA in a confirmation specific manner using residues in the recognition helix α3 and the β-wing through hydrogen bonds and van der Waals interactions between protein and DNA.[19-21] Zαs from different proteins can bind DNA and induce B-to-Z conversion with varied degrees due to the amino acid sequence variations.[4,10,18] Our mutational study suggests that hZβ_{ZBP1} use amino acids in the putative β-wing differently from other Zαs. The putative β-wing region of hZβ_{ZBP1} might juxtaposition to Z-DNA and stabilizes interactions with it through charge-to-charge interaction instead of van der Waals interaction. Despite of the lacking proline in the putative β-wing, hZβ_{ZBP1} may still adopt a similar fold to those of other Zαs by having K160 structurally substituted for the conserved proline(s) found in other Zαs.

Our study here would contribute to understanding of how Zα family proteins can obtain high affinities towards Z-DNA in various modes. Future X-ray crystallographic studies on the hZβ_{ZBP1}/Z-DNA complex will provide further insight into the hZβ_{ZBP1}-Z-DNA interaction in atomic details.

Experimental Section

Cloning, expression and purification. Zβ_{ZBP1} (aa. 103-166) from human ZBP1 was cloned into pET28a expression vector (Novagen) encoding an N-terminally His$_6$-tagged fusion protein as described previously.[4] The expression and purification of hZβ_{ZBP1} protein was essentially the same as described elsewhere.[4] Briefly, hZβ_{ZBP1} expression plasmid was transformed into *E. coli* strain BL21(DE3). Transformed bacteria were grown at 37 °C to absorbance of 0.6-0.8 at 600 nm, induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside and grown further for 3 hrs at 37 °C. The protein was partially purified using a Ni^{2+}-NTA column according to the manufacturer's instruction (Qiagen). The N-terminal His$_6$-tag was then removed by thrombin (Roche) and the intact protein was further purified using anion exchange chromatography. Protein purity was judged to be higher than 95% by SDS-PAGE, and the protein was concentrated to 2 mM in a buffer containing 10 mM Hepes (pH 7.5) and 50 mM NaCl, and stored at −70 °C prior to use.

Isothermal titration calorimeter (ITC). The d(CG)$_3$ oligonucleotide was purchased from Bioneer, resolved in TE buffer (10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA), annealed and purified by resource-Q column prior to use. ITC experiments were performed as follows: both protein and DNA were prepared in the assay buffer (10 mM Hepes, pH 7.5, 80 mM NaCl) using PD-10 desalting column (Amersham Pharmacia Biosciences). For a typical ITC experiment, *ca.* 30 aliquots of DNA (5 μL of 100 μM in strand concentration) was injected per shot into a sample cell containing purified protein (10 μM) at 3 min intervals to allow the sample cell in the instrument to return to baseline. The titration cell was kept at 25 °C and stirred continuously at 300 rpm. The data were analyzed to determine binding constants using ORIGIN software provided with the instrument.

Preparation of hZβ_{ZBP1} mutants. All site-directed mutagenesis reactions were carried out by PCR based-mutagenesis technique with appropriate primers and QuikChange® mutagenesis kit according to the manufacturer's instruction (Stratagene). Sequences of all the mutants were confirmed by DNA sequencing. Mutant proteins were purified essentially by the same method as described for the wild-type hZβ_{ZBP1}.

Circular dicroism (CD). B-to-Z conversion of substrate DNA was monitored by CD. To assess conversion activities of wild-type and mutant hZβ_{ZBP1} proteins by CD, we used annealed d(CG)$_6$ sequence as the substrate DNA. In each measurement, protein was added into a 2 mm quartz cell containing 50 μg/mL (25 μM in strand concentration) of

annealed $d(CG)_6$. The final concentration of the protein was adjusted to 30 μM so that the protein volume did not exceed 5% of the total volume. All CD spectra were measured in CD buffer (10 mM Hepes, pH 7.5, 50 mM NaCl and 0.1 mM EDTA) at 25 °C using Jasco J-810. For kinetic measurements, CD signal changes at 255 nm were recorded at an 1-sec interval up to 40 min.

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